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The Interleukin-4 Receptor Activates STAT5 by a Mechanism That Relies upon Common γ-Chain*

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Interleukin (IL)-4 signaling proceeds via cytoplasmic activation of the Janus kinases JAK1 and JAK3 and the signal transducer and activator of transcription STAT6. We show that the IL-4 receptor, like other cytokine receptor systems utilizing the common receptor γ-chain (γc), is also connected to a signaling pathway that involves STAT5. Both STAT5a and STAT5b become tyrosine-phosphorylated and acquire specific DNA-binding properties in response to IL-4 receptor stimulation in the murine pro-B cell line Ba/F3. In preactivated human T cells, STAT5 became activated in an IL-4-dependent fashion as assayed by IL-4-induced STAT5 translocation from the cytoplasm to the cell nucleus and by binding to cognate DNA. Moreover, stimulation of preactivated human T cells by IL-4 led to specific transcriptional upregulation of STAT5 target genes. IL-4 receptor-mediated STAT5 activation is dependent on the presence of γc and JAK3 within the receptor complex. In COS-7 cells, the JAK/STAT pathway leading from the IL-4 receptor to STAT5-dependent regulation of a reporter gene relied largely on coexpression of JAK3. In Ba/F3 cells, studies on signal transduction evoked by directed specific receptor homo- or heterodimerization revealed that STAT5 activation can be triggered exclusively by IL-4R heterodimers containing γc.

Interleukin (IL)-4,[1] a pleiotropic modulator of the immune system (1), exerts its activity on target cells through the interleukin-4 receptor. Different cell types, including lymphoid and myeloid blood cells, express a bipartite IL-4 receptor (IL-4R), which consists of the IL-4R α-chain (IL-4Rα) (2) and the common γ receptor chain (γc) (3). IL-4Rα may also form a functional IL-4 receptor in conjunction with the IL-13 receptor instead of γc, especially in nonimmune cells (4). *In vitro* IL-4Rα has also been shown to trigger intracellular signal transduction as a homodimer without participation of a heterologous receptor subunit (5, 6); however, the possible physiological relevance of IL-4Rα homodimers is not yet known.

IL-4-induced dimerization of receptor subunits results in the rapid onset of various cytoplasmic events. Janus kinases JAK1 and JAK3, which are associated with IL-4Rα and γc, respectively (7, 8), become activated, probably by transphosphorylation, and as a result several other constituents of the activated receptor complex are phosphorylated. Tyrosine phosphorylation of IL-4Rα, IRS-2 (insulin receptor substrate 2, originally termed 4PS/IL-4-induced phosphorylation substrate), phosphatidylinositol 3-kinase, STAT6, and probably other proteins generate a network of protein-protein contacts mediated by interactions between phosphotyrosines and Src homology 2 domains (9). Despite structure-function studies by several laboratories (10–16), it is only partially understood how these IL-4-induced molecular events lead to long term cellular processes such as suppression of apoptosis, proliferation, and differentiation.

The aspect of IL-4R-triggered intracellular signal transduction that has been best characterized is the JAK-STAT pathway. STAT (signal transducer and activator of transcription) factors are central components of signaling cascades triggered by cytokine receptors and are phosphorylated in response to ligand stimulation through receptor-associated Janus kinases (JAKs). STAT factors then dimerize and translocate to the cell nucleus, where they interact with cognate DNA sequences of γ-interferon-responsive sites (GASs) and modulate transcription of target genes (17).

Following signal induction by the ligand, both the IL-4 receptor and the IL-13 receptor specifically mediate the activation of STAT6 (15, 18–21). Gene regulation by STAT6 appears to be of central importance for IL-4-governed immune regulation, since STAT6 knockout mice are unable to develop Th2 cells; are impaired in cell surface expression of CD23, IL-4Rα, and major histocompatibility complex class II; and show a drastic defect in immunoglobulin class switching (22–24).

Results obtained in the course of investigations on related cytokine receptors raised the question of whether STAT6 is the only STAT protein involved in IL-4-induced intracellular signaling. The IL-4 receptor shares the common γ-subunit with the receptors for IL-2, IL-7, IL-9, and IL-15 (25), and all cytokine receptors utilizing the γc subunit, with the exception of the IL-4R, activate STAT5 in a ligand-inducible manner (26–34). Two forms of STAT5 have been described, STAT5a and STAT5b. STAT5a (first named mammary gland factor) was originally cloned and characterized as a regulator of the β-casein gene promoter and an intracellular mediator of prolactin action on mammary epithelial cells (35). STAT5b differs from STAT5a mainly in the carboxyl-terminal region, which is in-

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[1] The abbreviations used are: IL, interleukin; hIL, human interleukin; mIL, murine interleukin; IL-4R, interleukin-4 receptor; IL-4Rα, human interleukin-4 receptor α-chain; γc, common receptor γ-chain; JAK, Janus kinase; STAT, signal transducer and activator of transcription; GAS, γ-interferon-responsive site; DTAF, dichlorotriazinaminofluorescein; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pairs; PHA, phytohemagglutinin.

volved in transcriptional transactivation (36). It has been detected in mammary glands, hematopoietic cells, and liver tissue (31, 37–39). As well as being activated by the prolactin receptor and cytokine receptors of the "γc family," STAT5 is also involved in signaling by the IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor receptors. It also participates in cellular reactions triggered by the receptors for growth hormone, thrombopoietin, erythropoietin, and epidermal growth factor (37, 40–42). The fact that STAT5 activity is controlled by this wide variety of cytokines and growth factors in different cell types suggests an important role of STAT5 in the general regulation of cell differentiation and cell growth. A recent report provided evidence for IL-4-induced tyrosine phosphorylation of STAT5 in primary human B cells (43).

In this study, we investigated the responsiveness of STAT5 to ligand-induced activation of the IL-4 receptor in different cell systems. We demonstrate that STAT5 is activated in response to IL-4 stimulation in cell lines and in primary human cells. Upon IL-4 treatment of these cell types, STAT5 is tyrosine-phosphorylated and translocates to the cell nucleus, where it binds to a cognate DNA element and induces specific target genes.

A further goal of this work was to obtain information on the mechanisms that underlie IL-4R-mediated activation of STAT5. In Ba/F3 cells, IL-4-induced STAT5 activation was dependent on the contribution of the common receptor γ-chain. In COS-7 cells, expression of heterologous JAK3, the binding partner of γc, was crucial for full activation of STAT5 by the IL-4R.

EXPERIMENTAL PROCEDURES

DNA Constructs—Recombinant DNA work was performed according to standard procedures (44). Expression plasmids pKCR-4α and pKCR-pγ, encoding human IL-4Rα and human γc epitope tagged at the amino terminus, respectively, have been described (5, 45). Expression plasmids pXM-STAT5a encoding murine STAT5a (35, 36, 38, 46) and pXM-STAT6 encoding human STAT6 (47) were described elsewhere. Expression construct pcJAK3 (48) was kindly provided by C. Gurniak and L. Berg (Harvard University).

Cell Culture and Generation of Stably Transfected Cell Lines—Ba/F3-derived cell lines and COS-7 cells were cultured as described previously (36, 45, 49). Activated peripheral human T cells (prestimulated with PHA; PHA blasts) were obtained from healthy donors and prepared by centrifugation through Ficoll-Hypaque (50). Stable transfection of Ba/F3 cells with hIL-4 receptor constructs has been described in detail (5, 45). Generation and characterization of all Ba/F3-derived cell lines has been described (4, 45) with the exception of BAF-pγ and BAF-4α/γ-pγ/4α+pγ. These lines were obtained by stable transfection of Ba/F3 or BAF-4α/γ-pγ/4α (5) with the expression construct pKCR-pγ (45).

Immunoprecipitation and Immunoblotting—Immunoprecipitation and immunoblotting were carried out as described previously (5). Cleared cell lysates were incubated with 1 μl of rabbit serum-specific for STAT5a or STAT5b (51) for 3 h at 4 °C. Precipitation conditions employed for other antibodies were as described (5, 45, 52). Immunocomplexes were collected from lysates with 25 μl of protein A-Sepharose (Sigma) or anti-mouse IgG-agarose (Sigma) and analyzed by Western blot as described (45). The Western blots were detected using peroxidase-conjugated anti-Tyr(P) antibody RC20 (Transduction Laboratories) and anti-STAT5a or anti-STAT5b serum at a dilution of 1:20,000 (5, 48). Stripping of blots for reprobing was done by incubating the membranes with 0.1 M glycine, pH 2.9, at room temperature for 20 min followed by extended washing. For Western blot analysis of total Ba/F3 cell lysates, pellets equivalent to 3×10^5 cells were incubated with 2.5 units of Benzonase (Boehringer Mannheim) for 10 min at room temperature in protein sample buffer prior to gel loading. Whole cell extracts from transiently transfected COS-7 cells were prepared as described (36). 80 μg of total protein was applied per gel lane. For detection, rabbit serum against STAT5, STAT6, human JAK3 (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or a mouse monoclonal antibody against murine JAK3 (48) (a gift by C. Gurniak, Harvard University) was used.

Adsorption of Activated STAT5 to Solid Phase-coupled DNA—Samples of 3×10^7 starved Ba/F3-derived cells or prestimulated human peripheral T cells (50) were incubated at 37 °C for 10 min in 1 ml of medium containing no cytokine, 7 nM hIL-4, or 50 nM antibody P5D4. Whole-cell extracts were prepared by suspending the cells in 70 μl of a low salt buffer containing 20 mM HEPES, pH 7.9, 100 mM NaCl, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 100 μM Na_3VO_4 followed by three cycles of freeze-thawing and centrifugation at 4 °C for 30 min at $10,000 \times g$. Supernatants were incubated with 100 μg of M280-Streptavidin Dynabeads (Dynal) carrying multimers of oligonucleotides derived from the proximal STAT5-binding site (GAS element) of the bovine β-casein promoter (5′-GATCAGATTTCTAGGAATTCAATCC-3′) or from the STAT6-specific GAS element in the human immunoglobulin ε promoter (5′-GATCAACTTCCCAAGAACAGAA-3′). The oligonucleotides were annealed, ligated, biotinylated, and coupled to the beads according to the manufacturer's recommendations. Incubation of cell lysates in low salt buffer (see above) was for 1 h at room temperature. After three washes with low salt buffer, bound proteins were eluted from the beads with 50 mM sodium acetate, 1 M NaCl for 30 min at room temperature and subjected to SDS-polyacrylamide gel electrophoresis followed by Western blot analysis using antiserum against STAT5 or STAT6 for detection.

Immunofluorescence Microscopy—Samples of 10^5 starved BAF-4α-pγ cells (5) were left untreated or stimulated with mIL-3 or hIL-4 as described (45) for 30 min. Prestimulated human peripheral T cells (PHA blasts) were obtained as described (50), divided into samples of 10^5 cells, and then left untreated or incubated with 10 nM hIL-2 or hIL-4 for 30 min at 37 °C. Individual cell samples were suspended in 500 μl of ice-cold PBS and spun onto glass coverslips at $220 \times g$ for 3 min using a cytospin device (Hettich). Cells were fixed for 10 min in methanol at −20 °C and for 2 min in acetone at −20 °C. After incubation with 0.1% bovine serum albumin in PBS for 20 min at room temperature, cells were incubated with anti-STAT5a serum (5 μg/ml) and dichlorotriazinaminofluorescein (DTAF)-conjugated anti-rabbit IgG (Dianova) (0.6 μg/ml) for 60 min each. Nuclei were stained with Hoechst 33258 (10 μg/ml in phosphate-buffered saline) for 3 min. Slides were then examined in the epifluorescence mode using a Zeiss Axiophot microscope.

Transient Transfection of COS-7 Cells, Reporter Gene Assay—Transient transfection of COS-7 cells was performed by the calcium phosphate method as described (35). Generally, 2 μg of expression plasmid, 2 μg of luciferase reporter gene construct, and 1 μg of control plasmid pCH 110 were applied per individual transfection. β-Galactosidase and luciferase activity was determined as described (35). For cytokine stimulation experiments, COS-7 cells were treated with 7 nM of hIL-4 for 10 h and processed as described previously (53).

Electrophoretic Mobility Shift Assay—Whole cell extracts were prepared from untreated or cytokine-stimulated Ba/F3-derived cells, and band shift assays were performed as described (53). As a probe, the proximal STAT5-binding element 5′-AGATTTCTAGGAATTCAAATC-3′ from the bovine β-casein promoter was used. For specific antibody supershifting of DNA-protein complexes, 5–10 μg of specific antibody against STAT5a, STAT5b (51), or STAT6 (5) was added to the binding reactions.

RT-PCR Analysis for Induction of STAT5 Target Genes—BAF-4α-pγ cells and PHA blasts were prepared and stimulated with cytokines as described above. After cytokine treatment for 60 min at 37 °C, cells were subjected to isolation of total RNA as described (49). Reverse transcription was performed by using Moloney murine leukemia virus reverse transcriptase (Peqlab, Erlangen, Germany) and oligo-dT primer starting from 1 μg of RNA in a total volume of 20 μl according to the manufacturer's instructions. 3 μl of cDNA solution was subsequently used to amplify specific cDNAs by 30 cycles of the program 30 s at 95 °C, 30 s at 60 °C, and 1 min at 72 °C using PowerScript DNA Polymerase (PAN Systems, Nürnberg) following the supplier's protocol. 60 pmol of the respective specific primer was employed in each individual reaction, which was performed in a total volume of 30 μl. Specific primers were designed such that sequence similarity between murine and human mRNAs was maximal in the respective hybridization regions. The following primers were used: for *cis*, 5′-CTGCTGTGCATAGCCAAGACGTTC-3′ and 5′-CAGAGTTGGAAGGGGTACTGTCGG-3′; for *osm*, 5′-CGGCACAATATCCTCGGCATAAGG-3′ and 5′-TGCTCCTGGAAGGTCTGATTTTGC-3′; for *pim-1*, 5′-ACGTGGAGAAGGACCGGATTTCC-3′ and 5′-GATGTTTTCGTCCTTGATGTCGC-3′; and for *gapdh*, 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ and 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′. Sizes of the expected amplification products are 574 bp for *cis*, 387 bp for *osm*, 316 bp for *pim-1*, and 306 bp for *gapdh*. 15 μl of each reaction was analyzed by agarose gel electrophoresis.

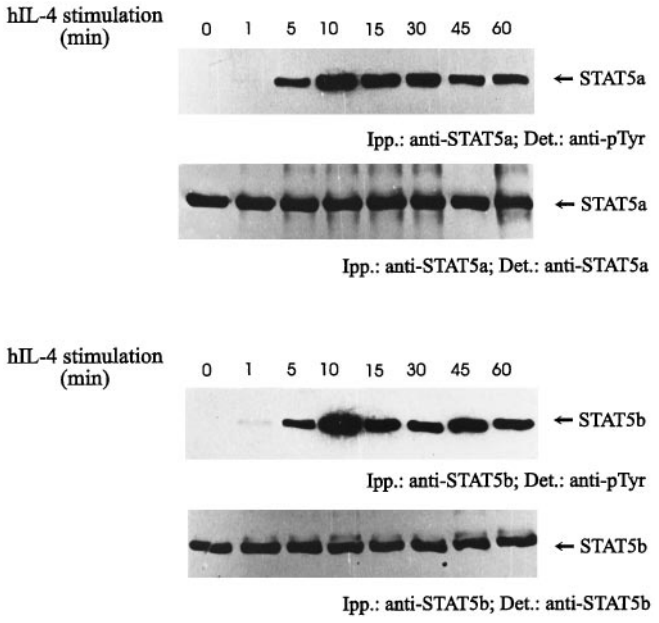

FIG. 1. **Tyrosine phosphorylation of STAT5a and STAT5b induced by human IL-4.** Starved BAF-4α-pγ cells expressing the bipartite hIL-4 receptor complex were stimulated with 7 nM hIL-4 for the times indicated. From cell lysates, STAT5a (*top*) or STAT5b (*bottom*) was immunoprecipitated (*Ipp.*). After Western blot, tyrosine phosphorylation was analyzed by detection (*Det.*) with anti-Tyr(P) antibody. To verify equal loading, the blots were reprobed with antibodies against STAT5a and STAT5b, respectively.

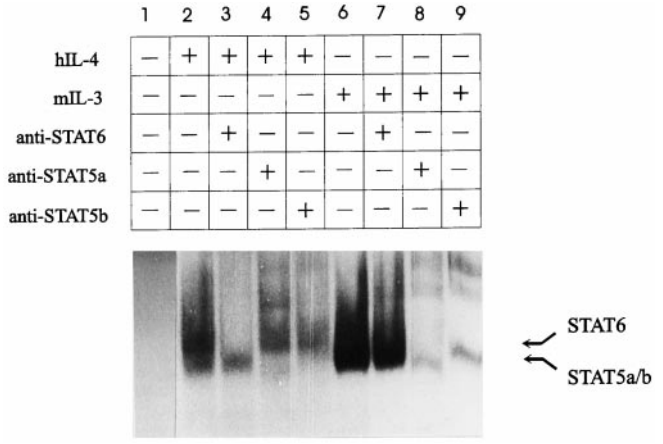

FIG. 2. **Induction of DNA binding activity of STATs in BAF-4α-pγ cells by cytokine stimulation.** After starvation or additional treatment with hIL-4 or mIL-3 for 30 min, cells were lysed and subjected to bandshift assays using the ^{32}P-labeled proximal STAT-binding element from the bovine β-casein promoter as a probe. Supershifting of protein-DNA complexes containing STAT6, STAT5a, or STAT5b, respectively, was achieved by adding specific antibodies to the binding reactions as indicated. The *arrows* on the *right* denote STAT6- and STAT5a/b-containing complexes.

RESULTS

The Human Interleukin-4 Receptor Mediates both Tyrosine Phosphorylation and Specific DNA Binding of STAT5a and STAT5b—The murine pro-B cell line BAF-4α-pγ stably expresses the bipartite human IL-4 receptor complex (5, 45). This cell line was used to address whether hIL-4-induced activation of the hIL-4R can affect the tyrosine phosphorylation state of STAT5. Fig. 1*A* shows that hIL-4 stimulation results in a rapid and profound tyrosine phosphorylation of both STAT5a and STAT5b, which reaches a plateau 10 min after cytokine treatment and is sustained for at least 60 min.

We next wished to demonstrate ligand-induced specific DNA binding of STAT5 in comparison with STAT6. BAF-4α-pγ cells were stimulated with hIL-4 or, as a control for STAT5 activation, mIL-3. Cell lysates were subjected to an electrophoretic mobility shift assay using the proximal STAT5 binding site from the bovine β-casein promoter as a DNA probe (Fig. 2). Both hIL-4 and mIL-3 evoked the formation of DNA-protein complexes (*lanes 2* and *6*). hIL-4 induced two distinct complexes of only slightly different migration behavior that could only be separated by relatively long gel runs (*lane 2*). Since we had previously found that the STAT5-binding DNA element from the β-casein promoter also interacts with phosphorylated STAT6 (47), it was necessary to determine the identity of the STAT proteins contained in the two hIL-4-induced complexes. Specific antibodies directed to STAT5a, STAT5b, or STAT6 were individually added to binding reactions in order to specifically supershift complexes containing the respective STATs. In extracts from hIL-4-stimulated cells, supershifting by anti-STAT6 did not affect the faster migrating complex (*lane 3*). This complex consisted of DNA-bound STAT5, since it was partially reactive with both anti-STAT5a (*lane 4*) and anti-STAT5b (*lane 5*). These antibodies were specific to the two forms of STAT5 and showed no cross-reactivity with STAT6. In contrast, the upper hIL-4-induced complex (*lane 3*) was affected selectively by anti-STAT6 but was not supershifted by the STAT5 antibodies, thus indicating that the upper complex contained STAT6. As expected, cell stimulation with mIL-3 did not yield STAT6-containing complexes (no supershift with anti-STAT6, *lane 7*) but evoked STAT5 complexes that could be partially supershifted with either anti-STAT5a (*lane 8*) or anti-STAT5b (*lane 9*).

IL-4 Induces Full and Specific Activity of STAT5 in Human Peripheral T Cells—In order to evaluate the general physiological significance of STAT5-activation through the hIL-4R, we investigated PHA blasts in comparison with BAF-4α-pγ.

First we addressed hIL-4-induced specific DNA binding of STAT5a and STAT5b. Both BAF-4α-pγ and human PHA blasts were incubated with hIL-4 and examined by DNA affinity absorption and immunoblot analysis for the presence of activated, DNA-binding STAT5a and STAT5b. Multimerized DNA elements derived from the proximal STAT5 binding site of the β-casein promoter ("GAS box") coupled to magnetic beads were used in this assay as a specific high affinity matrix. As shown in Fig. 3*A*, STAT5a as well as STAT5b bound to the STAT5 cognate DNA motif upon hIL-4 stimulation of both cell types. We also tested by immunoprecipitation experiments if IL-4-induced tyrosine phosphorylation of STAT5 and other signaling mediators is comparable in Ba/F cells and T cells (Fig. 3*B*). STAT5 as well as STAT6, JAK1, and JAK3 became tyrosine-phosphorylated in a hIL-4-dependent fashion.

The subcellular localization of STAT5 was then investigated by analyzing cytokine-induced translocation of STAT5 from the cytoplasm to the nucleus with fluorescence microscopy (Fig. 4). BAF-4α-pγ cells and human PHA blasts were stimulated with saturating concentrations of hIL-4, fixed, and incubated with antiserum against STAT5a. As positive controls, cells were stimulated with cytokines known to activate STAT5 in the respective cellular context (mIL-3 for BAF-4α-pγ, hIL-2 for human PHA blasts). Subsequently, STAT5a within cytokine-treated and -untreated cells was detected after staining with fluorescently labeled anti-rabbit IgG. In order to readily localize cell nuclei, all cell samples were stained with the dye Hoechst 33258 prior to microscopic inspection. Whereas in all unstimulated cells STAT5a-related fluorescence was maximal in the cellular periphery and thus in the cytoplasm, in cells treated with hIL-4 or control cytokines a clear accumulation of STAT5a in the nuclei was observed. These results indicate that

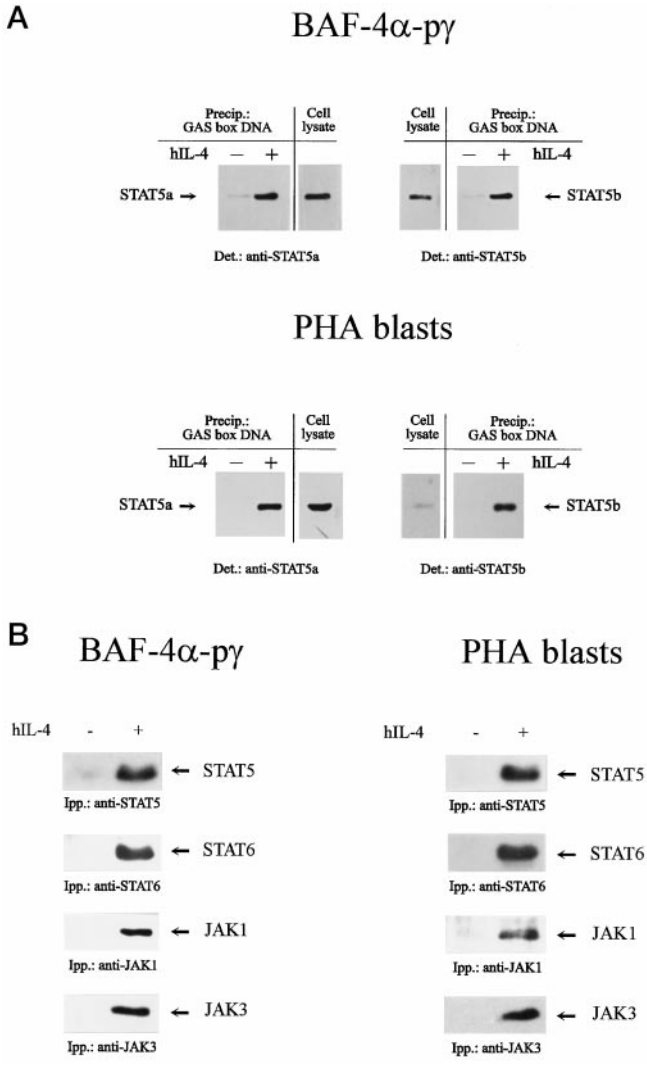

FIG. 3. *A*, hIL-4-induced activity of STAT5a and STAT5b analyzed by specific adsorption to cognate DNA. Starved BAF-4α-pγ cells (*top*) and human PHA blasts (*bottom*) were left untreated or stimulated with hIL-4 and lysed as described under "Experimental Procedures." Lysates were incubated with multimerized STAT5 binding DNA elements (β-casein promoter proximal STAT binding site, *GAS box DNA*) coupled to magnetic beads as described under "Experimental Procedures." Collected proteins were subjected to SDS-PAGE and Western blotting followed by detection (*Det.*) with specific antibodies to STAT5a (*left*) or STAT5b (*right*). Samples of total cell lysate were applied as controls for the specificity of Western blot detection. *B*, hIL-4-induced tyrosine phosphorylation of STAT5, STAT6, JAK1, and JAK3 analyzed by immunoprecipitation (*Ipp.*) and Western blot. Cells were treated as in *A* and subjected to lysis and precipitation with the indicated antibodies. Blots were probed with anti-Tyr(P).

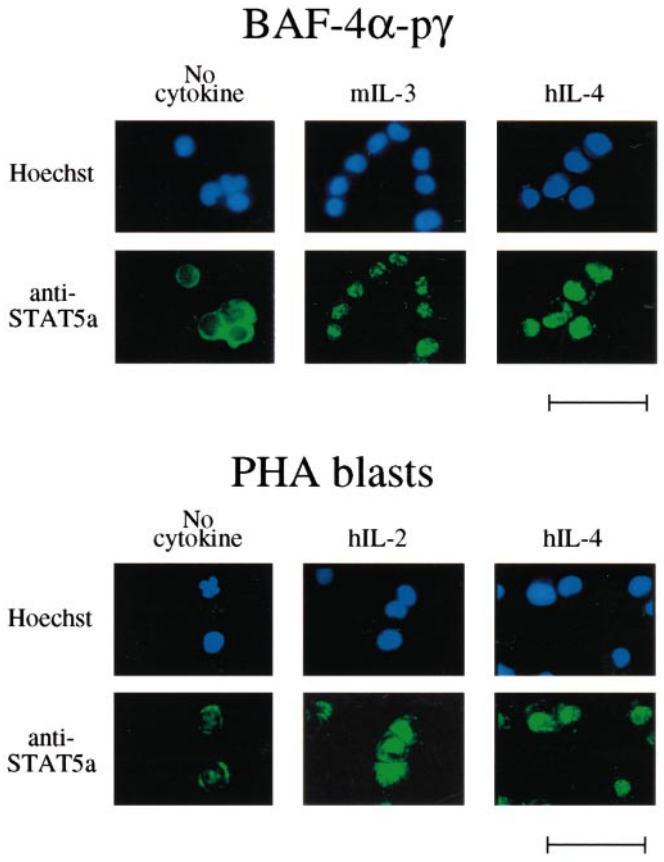

FIG. 4. **hIL-4-induced translocation of STAT5a to the cell nucleus.** Starved BAF-4α-pγ cells (*top*) and human PHA blasts (*bottom*) were either left untreated (*No cytokine*) or treated with the indicated cytokines for 30 min and fixed on glass coverslips. Cells were stained with anti-STAT5a serum plus DTAF-conjugated anti-rabbit IgG and, subsequently, with Hoechst 33258 as described under "Experimental Procedures." Hoechst 33258 staining of nuclei (*blue*) and fluorescence staining by anti-STAT5a/DTAF (*green*) was visualized by fluorescence microscopy. *Scale bar*, 100 μm.

stimulation of the hIL-4 receptor by ligand binding not only leads to tyrosine phosphorylation and dimerization of STAT5a but also renders this transcription factor fully capable of translocating to the nucleus and hence reaching its physiological site of action.

A further issue in the analysis of hIL-4-induced STAT5 activation was the question of STAT5-specific gene transcription. It has recently been shown that the transcriptional activity of certain genes, such as *cis* (54), *osm* (46), and *pim-1* (55), is specifically regulated via STAT5. We studied transcription of these genes in response to hIL-4 stimulation of BAF-4α-pγ cells and human PHA blasts by RT-PCR analysis of total RNA using STAT5-activating cytokines (mIL-3 for BAF-4α-pγ, hIL-2 for PHA blasts) as positive controls. As shown in Fig. 5, all three STAT5 target genes were not transcribed to any detectable level in cytokine-starved cells, whereas mRNA encoded by the "housekeeping" gene *gapdh* was clearly present. However, treatment of both BAF-4α-pγ and human PHA blasts with hIL-4 as well as with the respective control cytokines resulted in a profound expression of all three STAT5 target genes examined.

Taken together, these results clearly demonstrate that the IL-4 receptor is capable of exerting full activation of STAT5 in both transfected murine cells and in primary human T cells. IL-4-induced STAT5 activity extends from nuclear translocation to specific recognition of cognate DNA and transcriptional regulation of STAT5-responsive genes.

Recruitment of γc and JAK3 into the IL-4 Receptor Complex Is Mandatory for the Activation of STAT5—We have demonstrated previously that γc and Janus kinase JAK3 are dispensable for hIL-4R-mediated activation of STAT6 (5). We were therefore interested to learn if the same is also true for STAT5, and two different cellular systems were employed to address this question.

Recently, we showed that COS-7 cells do not express significant amounts of endogenous STAT5 or STAT6 but provide all components necessary for transiently expressed STATs to drive transcription of a suitable reporter gene construct (36, 47, 52, 53). Therefore, we reconstituted the bipartite hIL-4 receptor in COS-7 cells by transient expression of both hIL-4Rα and human γc and introduced a luciferase reporter gene under the control of the STAT5-responsive β-casein promoter (53). Sub-

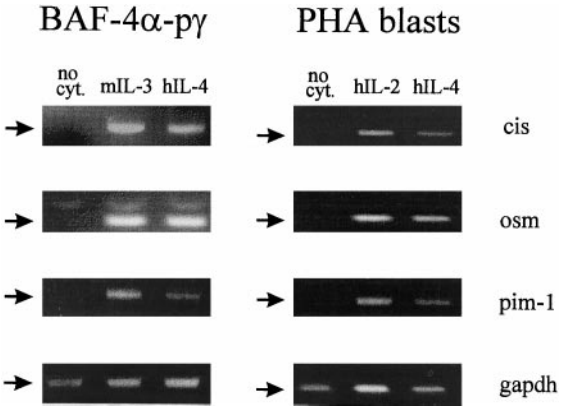

FIG. 5. **RT-PCR analysis of cytokine-induced STAT5 target gene expression in BAF-4α-pγ cells (*left*) and human PHA blasts (*right*).** Starved cells were left untreated (*no cyt.*) or incubated with the indicated cytokines for 60 min. Total RNA was extracted from cell samples, was transcribed into cDNA, and was amplified for the respective gene fragments using specific primers for *cis*, *osm*, *pim-1*, and *gapdh* as detailed under "Experimental Procedures." Amplification products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. The *arrows* indicate the specific DNA fragments of 574, 387, 316, and 306 bp, respectively. *gapdh* was included as a positive control.

sequently, hIL-4-dependent luciferase activity was determined (Fig. 6*A*).

In order to address the possible role of γc in the mediation of STAT5 activity, we tested the effect of additional expression of JAK3, which is not endogenously expressed in COS-7 cells (32). As a control, STAT6-triggered transcription from the β-casein promoter was measured, since we had shown previously that STAT6 also exerts hIL-4R-mediated transactivation activity in this experimental system (47). Both STAT6 (*column 4*) and STAT5a (*column 8*) triggered a strong transcription of the luciferase reporter gene upon ligand-induced activation of the hIL-4 receptor complex. A notable difference between the two STATs was observed when the contribution of heterologous JAK3 was assayed; whereas hIL-4 could evoke transcription through STAT6 to some extent even in the absence of JAK3 (*column 2*), STAT5a function in this assay was completely dependent upon the expression of JAK3 (*columns 6* and *8*). This finding strongly argues for an essential role of γc and JAK3 in the process of hIL-4-induced STAT5a activation. To further support this interpretation, we confirmed the expression of exogenous signaling factors in a parallel transfection experiment by Western blot analysis (Fig. 6*B*). Any mutual influence among these proteins on respective biosynthesis in COS-7 cells can be excluded, since we observed strong and equal expression of JAK3, STAT6, and STAT5a under all the conditions employed in the reporter gene experiment. Heterologous hIL-4R subunits, which were included as invariable constituents of all individual assays, could not be detected by Western blot analysis due to low expression and the small number of cells used in each transfection experiment. However, we can infer their expression indirectly, since the functional effects of dominant-negative mutants of both hIL-4Rα and human γc can be readily measured under these experimental conditions (52).

Finally, we addressed the contributions of the two hIL-4R subunits to ligand-induced activation of STAT5 in comparison with other signaling mediators. Ba/F3-derived cell lines that allow the experimental induction of homotypic *versus* heterotypic dimers of hIL-4R chains had been previously generated (5). We induced intracellular heterodimers of hIL-4Rα and γc or homodimers of either hIL-4Rα or γc. This was achieved by stimulation of engineered hIL-4R-derived receptor systems by specific agonists, *i.e.* hIL-4 or agonistic antibody P5D4 (Fig. 7*B*). Fig. 7*A* shows the human receptor constructs present in the tested cell lines. Defined dimerization events and control situations are depicted schematically; Ba/F3 cells (*I*) do not

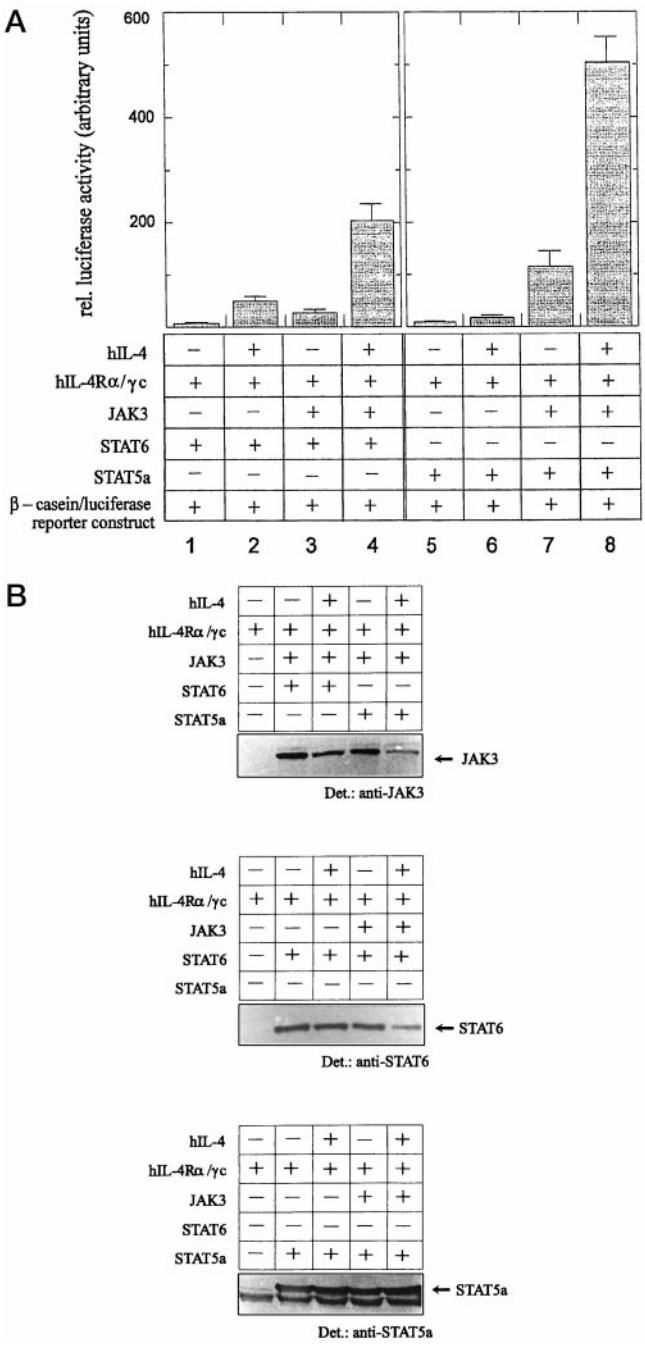

FIG. 6. **Reconstitution of hIL-4R-triggered transcriptional activity of STAT5 in COS-7 cells.** *A*, COS-7 cells were transiently transfected with expression constructs for both subunits of the hIL-4R, with a luciferase gene under transcriptional control of the β-casein-promoter, and with STAT6 (*left half*) or STAT5a (*right half*), respectively. An expression construct for murine JAK3 was cotransfected where indicated. The constitutively transcribed β-galactosidase gene was employed in parallel experiments to monitor transfection efficiency as described under "Experimental Procedures." Cells were left untreated or stimulated with 7 nM hIL-4 as indicated before luciferase activity was determined. Relative luciferase activity is expressed as the ratio of luciferase to galactosidase activity. Three individual experiments were carried out. *B*, expression of heterologous JAK3, STAT6, and STAT5a in transiently transfected COS-7 cells. Whole extracts of cells subjected to the indicated conditions of cytokine stimulation and transfection were prepared and analyzed by Western blot detection (*Det.*) using the antibodies indicated as described under "Experimental Procedures."

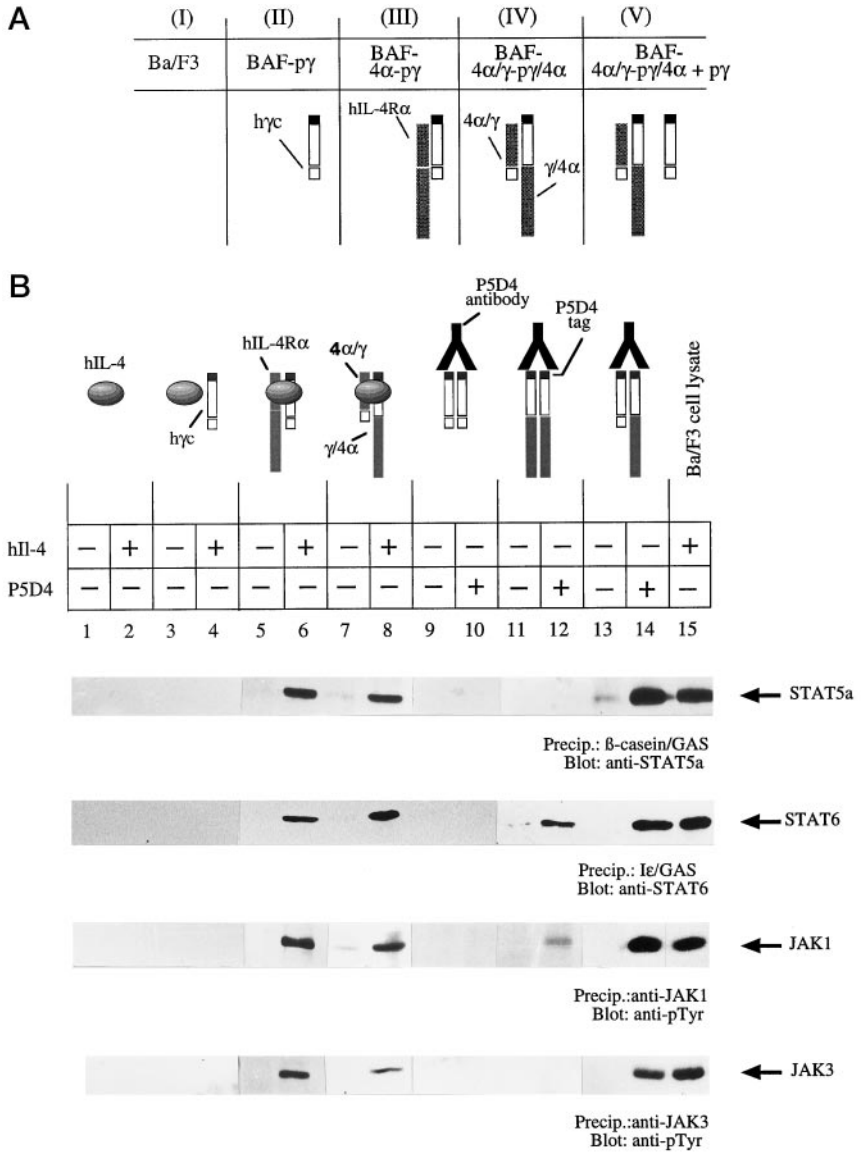

FIG. 7. **Effect of directed cytoplasmic homo- or heterodimerization of hIL-4R subunits on the activation of STAT5a.** *A*, hIL-4 receptor subunit composition in the cell lines used for the directed dimerization experiments and in controls shown in *B*. Ba/F3 cells contain no human receptor chain, and BAF-pγ cells carry human γc. BAF-4α-pγ cells express the bipartite hIL-4R complex with a P5D4 epitope tag fused to the amino terminus of γc; BAF-4α/γ-pγ/4α/γ cells express a bipartite hIL-4R complex with exchanged intracellular domains; BAF-4α/γ-pγ/4α+pγ cells contain a crossover hIL-4R complex plus an additional epitope-tagged γc chain. *B*, The intracellular dimerization situations depicted in the *upper part* of *B* were induced by treatment of Ba/F3-derived cell lines shown in *A* with 7 nM hIL-4 or 100 nM P5D4 antibody, respectively. For details, see text. Cells were lysed and then examined for stimulus-induced DNA-binding activity of STAT5a and STAT6 by specific adsorption to DNA using specific STAT binding DNA elements (see legend of Fig. 3 and "Experimental Procedures"). DNA-bound STATs were detected by Western blot analysis; total cell lysate of mIL-4-treated Ba/F3 cells served as a control (*lane 15*). Cells were also analyzed for tyrosine phosphorylation of JAK1 and JAK3 as in Fig. 3*B*.

contain any human IL-4 receptor component, BAF-pγ cells (*II*) express human γc, BAF-4α-pγ cells (*III*) are stably transfected with the authentic bipartite hIL-4R (45), BAF-4α/γ-pγ/4α cells (*IV*) express a hIL-4 receptor system with mutually exchanged intracellular domains (5), and cell line BAF-4α/γ-pγ/4α+pγ (*V*; this study) contains the "crossover receptor" pair plus an additional full-length human γc.

Fig. 7*B* shows the effect of intracellular hIL-4Rα homodimerization or hIL-4Rα/γc heterodimerization on the activity of STAT5a, STAT6, JAK1, and JAK3. Activation of STAT5a and STAT6 was analyzed by induced binding to specific cognate DNA elements (see "Experimental Procedures"), and activation of JAK1 and JAK3 was assayed by tyrosine phosphorylation of immunoprecipitates.

hIL-4 stimulation of Ba/F3 and BAF-pγ cells does not result in any detectable intracellular signaling (*lanes 1–4*), which was expected, since specific binding of hIL-4 is mediated only by the presence of the human IL-4Rα chain (2). Results obtained with a cell line that expresses hIL-4Rα as the only human receptor component are not included because they were indistinguishable from those with BAF-4α-pγ cells. The reason for this is the promiscuity of endogenous murine γc toward hIL-4 that has been discussed previously (45, 49).

Stimulation of BAF-4α-pγ (authentic receptor) as well as of BAF-4α/γ-pγ/4α cells (crossover receptor) with hIL-4 led to activation of STAT5a through α/γ heterodimerization (*lanes 5–8*). Homodimerization of cytoplasmic γc by cross-linking of epitope-tagged γc molecules did not result in STAT5a triggering (*lanes 9* and *10*). The DNA binding activity of STAT5a could be evoked by antibody P5D4 in 4α/γ-pγ/4α+pγ cells (*lanes 13* and *14*) in which cross-linking of extracellular epitope tags leads to intracellular α/γ heterodimerization.

Notably, the findings for homodimerized intracellular hIL-4Rα were clearly distinct when the effects on STAT5 and STAT6 were compared. As previously shown (5), antibody-directed homodimerization of the α-chain in BAF-4α/γ-pγ/4α cells can elicit a profound activation of STAT6 (*lanes 11* and *12*), whereas STAT5 activation occurs exclusively in response to hIL-4Rα/γc heterodimerization. STAT5 follows the pattern of JAK3 activation, which also requires the formation of heterodimers.

From these results, we conclude that mediation of STAT5a activity by the IL-4 receptor complex depends critically on the contribution of γc and JAK3.

DISCUSSION

The activated interleukin-4 receptor triggers the function of several intracellular signaling mediators. The best character-

ized IL-4-driven signal transduction cascade is the JAK/STAT pathway, which involves the IL-4- (and IL-13-) specific transcription factor STAT6. In this report, we have shown that the IL-4 receptor also connects ligand stimulation to STAT5 activation, a property shared by all other cytokine receptor systems comprising the γc.

Other laboratories have previously reported the inability of IL-4 to evoke the formation of DNA-protein complexes containing STAT5 (6, 27, 41, 55). We attribute our ability to demonstrate STAT5-DNA complexes mainly to two facts: (i) the availability of novel specific antisera directed to STAT5a and STAT5b (51) and (ii) the application of a pull-down assay that makes use of multimerized STAT-binding DNA oligomers coupled to magnetobeads. The sensitivity of this procedure is probably due to the involvement of multimeric clustered interaction targets for STATs, which provide a high local concentration of binding sites. This situation resembles the organization of the native β-casein promoter, which contains two adjacent STAT5 binding elements (35) and allows the formation of tetrameric STAT5 complexes in contact to the DNA (36), thus resulting in a particularly stable assembly.

One part of this study was concerned with the contribution of hIL-4R subunits to signal transduction via STAT5. We and others have recently observed that homodimers of hIL-4Rα can mediate intracellular signal transduction (5, 6). This unexpected finding raised the question of which specific roles, if any, γc and JAK3 have in IL-4-triggered signaling. To approach this problem, we employed two different cellular model systems that allowed us to study hIL-4R-mediated signaling and to simultaneously manipulate either the presence or absence of JAK and STAT factors or the composition of IL-4R subunit assembly.

Both experimental setups indicated an important role of γc and JAK3 in ligand-induced mediation of STAT5 function. This is consistent with the ability of all cytokine receptor systems, including the IL-4R, to use the γc to control the activity of STAT5. In the IL-4 receptor complex, activation of STAT5 is achieved by a different mechanism from the one that operates in STAT6 activation. STAT6 can be rendered active not only by experimentally induced homodimers of hIL-4Rα (5, 6) but also by the IL-4 receptor of human B cells that lack JAK3 (56). In contrast, triggering of STAT5, as shown in this report, requires the contribution of γc and JAK3. This finding is in line with data published by others who reported the strict requirement for JAK3 in IL-2 receptor-triggered STAT5 activation (32) and does not support the view that the IL-4Rα subunit is the only determinant of signaling specificity. Phosphorylation of IL-4Rα via γc/JAK3 and JAK1 or, alternatively, via homotypic juxtaposition of IL-4Rα/JAK1 cannot independently direct the whole set of cytokine-specific downstream reactions (6, 57). Our results attribute an essential role to the common γ-chain and its associated Janus kinase in the onset of signal transduction events involving STAT5.

The molecular details of STAT5 activation through the hIL-4R complex are still unknown. Its dependence on γc and JAK3 was not found to be mirrored by protein-protein associations detectable under our experimental conditions, but such interactions probably occur only transiently during the process of STAT5 phosphorylation and activation. We observed, however, a strong interaction between JAK1 and STAT5, which occurred independently of receptor stimulation[2] and supports a recent report that identified STAT5 as a binding partner for JAK kinases in a two-hybrid screen (58). Since some cytokine receptors (*e.g.* the interferon receptors) signal via JAK1 (25) but apparently do not activate STAT5, there must be determinants other than the contact between JAK1 and STAT5 that restrict STAT5 activation to particular receptor systems. Results obtained in this study make it tempting to speculate that γc may contribute to STAT5 specificity. A recent report showed that γc can influence signal transduction reactions such as phosphorylation of the IL-2 receptor β-chain and the tyrosine phosphatase SHP-2 by a mechanism that does not rely on JAK3 activity (59). Together with our findings, these results suggest a more complex role of γc in cytokine receptor signaling than was previously assumed. In the IL-5 receptor, activation of STAT5 strictly requires a specific proline residue in the cytoplasmic domain of the short α-subunit, which in the IL-5 receptor system might play a role that is functionally analogous to γc in the IL-4R complex (60).

Importantly, STAT5 activation through the human IL-4 receptor was not only observed in transfected cell lines but also in primary IL-4-reactive human T cells. Lymphoid cells are important target cells for cytokines that utilize the common γ-chain. STAT5 appears to be a common feature in signaling by these cytokines. However, to understand the implications of this, it will be important to establish the precise influence of STAT5 on cell physiology and cell function. We do not yet know what the relative "strength" of IL-4-induced signaling via STAT5 compared with reactions evoked by other cytokines is. In addition to relevant studies on STAT5-deficient animals (see below), it will be necessary to develop more quantitative assays to evaluate the physiological importance of the IL-4/STAT5 pathway.

Most IL-4-specific responses of immune tissues (*e.g.* Th2 cell development, immunoglobulin class switch to IgE) clearly coincide with STAT6 function (22–24). STAT5 may participate, however, in less characteristic and less obvious aspects of IL-4R signaling. It has been shown that STAT5b is involved in IL-3-induced proliferation of Ba/F3 cells (55), and in our hands a dominant-negative mutant of STAT5b can exert a similar, although less pronounced, effect on IL-4-evoked Ba/F3 proliferation.[3] A role for STAT5b in IL-4R-mediated DNA synthesis in certain cell types, perhaps in a synergistic mode with IRS-2, would therefore be conceivable. On the other hand, STAT5 may also play a role in IL-4-induced differentiation in a cell type-specific manner. It is interesting in this context that we did not readily observe IL-4-induced STAT5 binding to DNA in mammary epithelial cells (47). While it is possible that the novel antibodies and improved specific experimentation detailed in this report would still reveal some degree of IL-4-dependent STAT5 activation in such cells, this particular signaling pathway could also be operative only in certain tissues. Further analysis of different cell types and the application of sensitive assays will be necessary to clarify this issue. Since STAT5 target genes such as *cis* (54), *osm* (46), and *pim-1* (55) can be regulated in response to IL-4, it will be a challenge to understand which roles their gene products play in given cellular environments. A profound IL-4-induced up-regulation of the *cis* message in bone marrow was reported (61), arguing for a possible involvement of STAT5 in IL-4-controlled processes during hematopoiesis. Very recently, the phenotype of STAT5a/b knockout mice was reported (62), which showed STAT5 deficiency to be mainly associated with the physiological functions of growth hormone and prolactin. However, STAT5a/b knockout animals were also reported to show a decrease in the white cell count and severe defects in peripheral T cell proliferation. It will be very interesting to investigate in detail to what extent IL-4-induced reactions in T cells and other lymphoid cell types are affected.

[2] I. Erhardt, A. Lischke, and K. Friedrich, manuscript in preparation.

[3] K. Friedrich, W. Kammer, I. Erhardt, S. Brändlein, and R. Moriggl, manuscript in preparation.

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